

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 January 2003 (30.01.2003)

PCT

(10) International Publication Number
WO 03/008650 A1

(51) International Patent Classification⁷: C14C 3/28

(21) International Application Number: PCT/KR01/01942

(22) International Filing Date:
14 November 2001 (14.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2001-42627 14 July 2001 (14.07.2001) KR
2001-45205 26 July 2001 (26.07.2001) KR

(71) Applicant (for all designated States except US): **INSECT BIOTECH CO., LTD.** [KR/KR]; #116 Bioventure Center,, Korea Research Institute of Bioscience and Biotechnology, 52 Oun-dong, 305-333 Taejeon-si (KR).

(72) Inventors; and

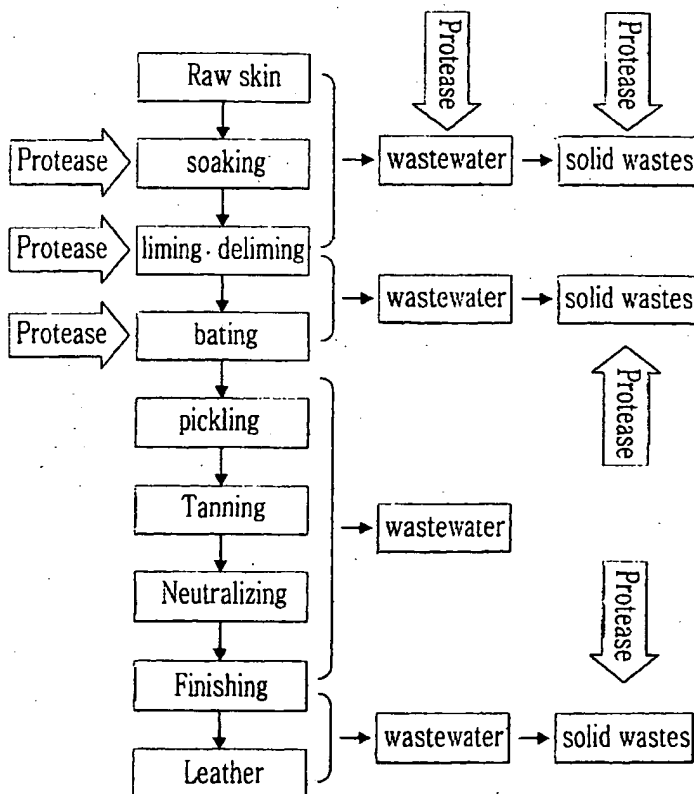
(75) Inventors/Applicants (for US only): **PARK, Ho-Yong** [KR/KR]; #12-403 Shindonga Apt., 1 Yongjon-dong, Dong-ku, 300-823 Taejeon-si (KR). **SON, Kwang-Hee** [KR/KR]; #103-1702 Hanbit Apt., Oun-dong, Yusung-ku, 305-755 Taejeon-si (KR). **KWON, Yong-Kook** [KR/KR]; #126-1307 Hanbit Apt., Oun-dong, Yusung-ku, 305-755 Taejeon-si (KR). **SHIN, Dong-Ha** [KR/KR]; 126-8, Shinsung-dong, Yusung-ku, 305-345 Taejeon-si (KR). **MIN, Sung-Gi** [KR/KR]; #1907-1001 Shinan Apt., Sang-dong, Wonmi-ku, 420-030 Buchon-si, Kyungki-do (KR).

(74) Agent: **LEE, Won-Hee**; 8th Fl., Sungji Heights II, 642-16, Yoksam-dong, Kangnam-ku, P35-080 Seoul (KR).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,

[Continued on next page]

(54) Title: METHOD FOR PREPARING LEATHER USING PROTEASE AND METHOD FOR TREATING WASTES DERIVED FROM LEATHER PROCESSING USING THE SAME



(57) Abstract: The present invention relates to a method for preparing leather using a protease and a method for treating wastes derived from leather processing using the same, which are advantageous in terms of preparation of leather of excellent quality, decreased waste production by reducing the amount of chemicals, and treatment or recycling of wastes in an environmentally friendly manner. The protease HY-3 produced from *Aranicola proteolyticus* HY-3 strain is added to the steps of soaking, liming, deliming and bating, among leather processing, and thus the leather is prepared. Additionally, the protease HY-3 is added to wastewater and solid wastes generated during the soaking and liming steps, and solid wastes generated during the liming, deliming, bating and finishing steps, so that the wastes are treated.

WO 03/008650 A1



MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK,
SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA,
ZW.

CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

Published:

— with international search report

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD FOR PREPARING LEATHER USING PROTEASE AND METHOD FOR
TREATING WASTES DERIVED FROM LEATHER PROCESSING USING THE
SAME

5

FIELD OF THE INVENTION

The present invention relates to a method for preparing leather using protease HY-3 and a method for treating wastes derived from leather processing using the same. More particularly, the present invention is concerned with a method for preparing leather, in which protease HY-3 produced from *Aranicola proteolyticus* HY-3 strain is added to the steps of soaking, liming, deliming and bating; and a method for treating wastewater and solid wastes derived from soaking and liming steps, and solid wastes derived from liming, deliming, bating and finishing steps, using the protease HY-3, having advantages of preparing leather of excellent quality, and environmentally friendly treatment or recycling of wastes.

20

BACKGROUND OF THE INVENTION

In general, the leather industry, as raw skins processing industry requiring expensive equipment and technique, is the chief industry determining quality of leather articles, such as shoes, bags, clothes and belts. In the leather industry, while the skins or hides of various

animals are subjected to complicated leather preparation processes, final product, that is, leather, is produced. Leather preparation processes using physicochemical and biological procedures are classified into water-requiring
5 wet processes and dry processes.

The wet process comprises the steps of brine curing for preventing putrefaction of raw skins by bacteria or molds, soaking for removing needless components of raw skins, liming for producing limed pelt, a deliming for removing
10 lime, bating for biologically treating unnecessary components, pickling for decreasing pH, neutralization, retanning, dyeing and fatliquoring.

More specifically, in the soaking step, large quantities of unnecessary proteins attached to raw skins or
15 hides, and salts or dirt, are removed. In the liming step, hairs and nonessential proteins are removed using lime. In the deliming step, the lime used in the previous step is removed. In the bating step for intensive surface cleaning, unnecessary proteins are removed. A hair saving method,
20 which is an environmentally friendly leather preparation method, uses a proteolytic enzyme for the liming and deliming steps. The liming step, among leather preparation processes, largely affects COD (chemical oxygen demand).

Though being mainly a light industry, the leather
25 industry is a representative pollution generating industry because of causing water pollution and soil pollution by large quantities of wastewater and solid wastes. Most

chemicals used in each step are discharged as wastewater after their use in wet processes.

In order to manufacture leather articles from raw skins, large amounts of chemicals are utilized. Additionally, use is made of large amounts of water, activators, limes, sulfides, salts, acids, chrome, synthetic tannin, dyes, fatliquoring reagents, binders, supplemental agents, brighteners and solvents. However, by overuse of sulfides and alkalis required for removal of various non-structural proteins in raw skins at the liming step, wastewater pollution becomes serious.

In the wastewater produced from the leather preparation, large amounts of organic and inorganic matters, such as high concentrations of salts-including sulfides and organic matters are contained, thus pollution levels being increased. Additionally, upon leather preparation, the liming, deliming, tanning and dyeing steps require large amounts of water and thus the used water is discharged as wastewater. The discharged wastewater, which contains hide scraps, hairs, soluble proteins and intermediate degradation products with high pollution load, flows into a wastewater reservoir. Such wastewater is characterized in that the highly toxic heavy metal, chrome is present and BOD (biological oxygen demand) and COD are very high, attributed to large quantities of organic and inorganic matters, and floatable substances.

In order to purify the wastewater, various equipment

and reagents are demanded and thus high wastewater-treating cost is incurred. Further, various solid wastes of the skins, such as fleshing scrap, pelt scrap, shaving scrap, trimming scrap, generated from the leather preparation processes, equal 50 % or more of the weight of the raw skins. The treatment cost for such solid wastes increases production costs.

SUMMARY OF THE INVENTION

10

Leading to the present invention, the intensive and thorough research into a leather preparation method and a waste-treatment method, carried out by the present inventors aiming to avoid the problems encountered in the prior arts, resulted in the finding that, using proteolytic enzymes, leather is prepared and wastes are treated, whereby leather of superior quality can be obtained, and the wastes from leather processing can be decreased in their amounts and be recycled.

20

Accordingly, it is an object of the present invention to provide a method for preparing leather using protease HY-3 produced by *Aranicola proteolyticus* HY-3 strain.

It is another object of the present invention to provide a method for treating wastes derived from leather processing using protease HY-3 produced by *Aranicola proteolyticus* HY-3 strain.

In an aspect of the present invention, there is

provided a method for preparing leather using protease HY-3 produced by *Aranicola proteolyticus* HY-3 strain.

In another aspect of the present invention, there is provided a method for treating wastes from leather preparation process using protease HY-3 produced by *Aranicola proteolyticus* HY-3 strain.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 is a schematic block diagram showing a preparation process of leather, capable of using protease HY-3 of the present invention.

Fig. 2 is a graph showing protein amounts eluted from leather upon use of protease HY-3 of the present invention;

15 ●:control ■:supernatant ▲:concentrated supernatant

DETAILED DESCRIPTION OF THE INVENTION

The present invention pertains to a method for preparing leather by use of protease HY-3 produced from *Aranicola proteolyticus* HY-3 strain.

The method of the present invention comprises the steps of soaking, liming, deliming and bating (see Fig. 1).

As for the protease HY-3 used for the leather preparation method of the present invention, microorganisms are separated from *Aranicola proteolyticus* HY-3 strains-cultured medium and thus the remaining enzyme-containing

supernatant is used, or a mixture of the protease HY-3 and an additive for increasing stability of the enzyme or a material introduced to each step, formulated in a preparation, is used.

5 The protease HY-3 of the present invention is preferably added at an amount of 0.1-15 wt% to the steps of soaking, liming, deliming and bating, and is the microorganism-removed enzyme-containing liquid from microorganism culture medium, or may be the preparation
10 mixed with an additive for increasing stability of the enzyme or a material introduced to each step.

 The present protease HY-3 has a maximum activity at 37 °C with a relative activity of 75 % or more at 20-40 °C. In addition, the protease shows a maximum activity at pH 8.0
15 and a relative activity of 80 % or more at pH 7.0-9.5 (Korean Pat. Application No. 2000-5479). Generally, soaking, liming and bating steps are carried out at 25-35 °C and at pH 8-9, so that proteins in the skins can be stably decomposed using the protease HY-3. Also, when large
20 quantities of salts are added to the steps of soaking, liming, deliming and bating, most proteolytic enzymes are decreased in their activity, whereas the present protease HY-3 maintains its activity, even at high salinity of 10 %, and thus can be applied to each step.

25 By using the protease HY-3, conventionally used organic and inorganic chemicals for leather preparation can be drastically decreased in their amounts, and thus

environmentally neutral leather-processing can be performed.

Hence, such protease HY-3 can be applied to leather processing, and in particular, be useful in removal of epidermis, hairs and soluble proteins through the steps of
5 soaking, liming, deliming and bating.

In the soaking step, when animal skins or hides subjected to salting treatment are delivered to a leather factory, water should be sufficiently added in a paddle or drum for removing various dirt and unnecessary components,
10 after which water absorbed into skins tissues over a long period of time allows the skins tissues to be restored to the normal skins softness of a live animal. So, unnecessary soluble proteins, dirt and hairs attached to raw skins or hides can be decomposed and then removed by treatment of
15 the protease HY-3. Through such procedure, the skin tissues become smooth and a next liming step can be easily conducted.

In the liming and deliming steps, which strongly affect COD in wastewater generated from the leather
20 processing, the protease HY-3 can decompose cells of stratum germinativum or base cells of hair root, using a principle of decomposing not the cortex but the medulla of hairs. Additionally, hair roots and hair follicles are removed, and thus the trichopore is certainly swelled, and
25 then hairs are eliminated, so that lime and other chemicals can be easily penetrated and thus treatment amounts of chemicals can be reduced.

In the bating step, the protease HY-3 removes unnecessary proteins in raw skins and loosens skin tissues, whereby chemicals for leather processing to be supplied after the bating step can be readily penetrated into the tissues and a bonding strength between chemicals and tissues is increased. Therefore, the required amounts of such chemicals are decreased, and increase of COD and BOD attributed to unbonded chemicals in wastewater is restrained. By use of the protease HY-3, soft and pliable leather can be produced and pollutants on the skins can be removed. Thereby, upon dyeing, the color is improved in dyeing evenness, and thus dyes are decreased in their amounts and leather goods are greatly increased in their quality.

15 In another embodiment of the present invention, a method for treating wastes derived from leather preparation process using the protease HY-3 is provided.

The wastes produced from the steps of soaking, liming, deliming, bating and finishing are treated by use of protease HY-3 of the present invention (see, Fig. 1).

20 The wastes may be formed in liquid or solid state, and also be recycled.

As protease HY-3 applicable to the leather preparation of the present invention, use is made of a microorganism culture medium, an enzyme-containing liquid remaining after the microorganism is separated from the above medium, or a formulated preparation mixed with an additive for

increasing stability of the enzyme or a material introduced to each step.

For treatment of the wastes derived from leather preparation process, one selected from the group consisting of a lipase and an amylase is used, together with the protease HY-3.

In order to treat the wastes derived from the leather preparation process, the protease HY-3 producing microorganism medium, the enzyme-containing liquid remaining after microorganism is separated from the above medium, or the formulated preparation mixed with the additive for increasing stability of the enzyme or the material introduced to each step is added to wastewater and solid wastes generated during the soaking and liming steps, solid wastes generated during the bating step, and solid wastes after the finishing step.

The wastes generated from the leather-preparation amount to 40-70 wt% of the initial weights of raw skins or hides. Most discharged solid wastes are classified as industrial wastes and thus burned up or simply buried. Hence, treatment cost of such solid wastes is considerably high in light of production cost.

The solid wastes, treatable by the protease HY-3, are exemplified by fleshing scraps, trimming scraps and hairs from the raw skins, generated during the soaking and liming steps; pelt scraps, after the bating step; and skin scraps, generated from the finishing step following a final drying.

In fleshing scraps and trimming scraps, proteins and lipids are not completely separated and are present in a mixed state, in which lipid component amounts to 30-50 % of total components. To fleshing scraps and trimming scraps, at least one enzyme selected from protease HY-3, lipase or amylase may be added.

The solid wastes, such as pelt scraps which are produced from the bating step after the liming step, comprise about 40 % of total wastes generated from leather processing. The pelt scrap comprises 4.0 % lipid, 1.5 % calcium, 5.5 % ash, 50-55 % water and 35-40 % protein. The protease HY-3 of the present invention, usable as a metallic protease, is increased in its activity when metal ions are present. The activity of the protease HY-3 is increased about 1.5 times in the presence of 1 mM calcium ions, and 1.2-1.4 times in the presence of other metal ions. Metal ions, such as calcium ions, are present in large amounts in the pelt scrap, so that the present protease HY-3 is effective for decomposition of protein in the pelt scraps.

To prepare leather goods from raw skins, large quantities of chemicals should be used. Also, water, activators, limes, sulfides, salts, acids, chrome, synthetic tannin, dyes, fatliquoring agents, binders, supplemental agents, brighteners, and solvents are employed. Overuse of sulfides and alkalis required for removal of various non-structural proteins in raw skins at the liming

step causes serious wastewater pollution. The wastewater discharged from the leather processing facilities includes organic, inorganic and floatable matters, and is characterized in that BOD and COD in wastewater is very high and chrome, a heavy metal having high toxicity, is present. By using the protease HY-3, the amounts of activators, limes, sulfides, salts, acids and chrome can be decreased.

Meanwhile, recycling of the wastes using a proteolytic enzyme is reported (Korean Pat. No. 1994-0007333), however, decomposition of proteins in the wastes using a protease-producing microorganism has not been reported yet. The microorganism of the present invention thrives even in the presence of a small amount of carbon source and nitrogen source, and protease HY-3 secreted from such microorganism can decompose the solid wastes derived from the leather processing. Using the protease HY-3, proteins in the wastes can be hydrolyzed to peptides to make foods, cosmetics and industrial products. Additionally, such protease can be used for saline-containing wastewater generated during the soaking and liming steps, and thus proteins in the wastewater can be recycled by decomposition of protease HY-3.

Further, waste leather can be utilized as feed and edible gelatin by cleavage of polypeptide chains in collagen molecules. By using the protease HY-3 for treatment of solid leather wastes, environmental pollution

is prevented and secondary products can be obtained.

EXAMPLES

5 A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

10 EXAMPLE 1 : Production of Protease HY-3

 In order to produce protease HY-3, a standard medium for growth of microorganism was sterilized under high pressure at 121 °C for 20 minutes, and then protease HY-3
15 producing *Aranicola proteolyticus* HY-3 strain (KCTC 0268BP) was added in the amount of 0.1-5 vol% on the basis of the whole volumes of the medium and cultured at 25-30 °C for 25-30 hours. The medium was subjected to membrane filtration and thus supernatant was separated from the
20 biomass. As necessary, the supernatant was concentrated 3-10 times through 10 kDa membrane filtration.

EXAMPLE 2 : Analysis of Protein Eluted From Liming and Deliming Steps Using Protease HY-3

25

 In order to investigate the effect of protease HY-3 at liming and deliming steps greatly affecting COD, the

following procedure was carried out.

In specifically, 20 g of skin was cut to suitable sizes, and introduced into 50 ml tubes, and then 10 ml of water was added thereto. Thereafter, 0.5 % sodium bisulfite and 0.5 % ammonium sulfate were added and reacted at room temperature for 15 minutes. After reaction, 0.2 % detergent and 0.5 % degreasing agent were added to the resulted solution and reacted at room temperature for 30 minutes. The three tubes were divided into a control containing no protease HY-3, and supernatant and concentrated supernatant prepared as in the above example 1. Each of tubes was allowed to stand at room temperature, and samples were obtained. Assay of protein eluted from the samples was performed using a protein assay kit (Bio-rad, USA) of modified Bradford method (Bradford, M., *Anal. Biochem.*, 1976, 72, 248).

From the experimental results, it can be seen that the protease HY-3 containing tubes have more eluted proteins, compared to the control, and also much more proteins are eluted by the concentrated supernatant than by the unconcentrated supernatant of microorganism-cultured medium.

EXAMPLE 3 : Use of Protease HY-3 at Bating Step

25

In order to increase stability and performance of the protease HY-3 in leather processing, the protease was

formulated in a preparation and thus used at the bating step.

Lyophilized protease HY-3 of the present invention was added in the amount of 0.5-10 wt% to 40-50 % ammonium chloride (NH_4Cl), 40-50 % ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), 0.005-0.01 % calcium chloride (CaCl_2) and 0.025-0.1 % lactose. To examine the bating effect of the formulated protease HY-3, the skins were subjected to deliming step, and then to bating step using different enzyme preparations, after which the skins were observed as to their surface state. In addition, the skins were subjected to tanning and finishing steps, and then the surface state was observed. As the protease preparation used in the bating step for comparison, use was made of Amoron (Chungmu Fermentation, Korea) and Oropon K (TFL, Germany). The experiment procedure is summarized in Table 1, below.

TABLE 1

Step	Chemicals	%	PH	Time (min)
Deliming	H_2O	200		60
	Deslon NAT (TEXAPEL)	0.2		
	Deliming Agent	3		
Bating	Lactic Acid	0.2	7.6-7.8	20
	Bating Agent	0.1-15		60
Pickling	H_2O	150	2.8-3.0	10
	NaCl	8		
	HCOOH (1:10)	0.5		20
	HCOOH (1:10)	0.5		20
	H_2SO_4	0.8		100
				Overnight
Tanning	Cr	3	3.6-3.8	60
	Cr	3		60
	Na_2CO_3	0.3		20

	Na ₂ CO ₃	0.3		20
	Na ₂ CO ₃	0.4		540
	Natural Dry			

The present protease HY-3 and controls (Amoron and Oropon K) were added at the bating step. More specifically, at the deliming step, chemicals shown in the above table 1 were added for 1 hour and then 0.2 wt% lactic acid and 0.1-15 wt% protease were added at the bating step. The bating step was carried out for 80 minutes, and then the pickling step for treating with saline matter-containing strong acid chemicals was carried out for 12 hours or longer and the chrome-containing tanning step was performed for about 12 hours, followed by drying the skins. Thereafter, the skins were observed.

To compare the effect of the used enzyme preparation at each step, the skins were subjected to deliming, bating and pickling steps while using the different enzyme preparations only in the bating step. The skins after bating were observed and the skins of wet-blue state were analyzed.

After bating, the skins treated by the formulated protease HY-3 are clearer and smoother in grain and surface of perioplie corium, compared to controls. As for wet-blue grain, the skins treated by the protease HY-3 are softer on their surfaces and higher in whiteness.

25 COMPARATIVE EXAMPLE 1 : Comparison of Properties of Leather

Prepared By Adding Deliming Agent at Bating Step

In order to investigate the activity of protease HY-3 in the presence of different deliming agents, this example was performed in the same manner as in the above table 1, except that each of deliming agents, such as 3 wt% ammonium chloride (NH_4Cl), 3 wt% ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), and 2 wt% ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) and 1 wt% ammonium chloride (NH_4Cl), was added in the bating step, and then the protease HY-3 was added thereto. The physical properties of the skins were compared for addition of each deliming agent.

Generally, a deliming agent is added to increase the effect of bating. Upon addition of deliming agent, the effect of protease HY-3 by such deliming agent was examined.

1-1 Addition of Ammonium Chloride

While performing the same procedure as in the above table 1, 3 wt% of ammonium chloride (NH_4Cl) was added at the bating step and stirred for 60 minutes. Additionally, each of protease HY-3, Amoron and Oropon K was added. The rawskin was cut to width 30 cm x length 20 cm. As a control, no deliming agent was used at the bating step.

Thusly obtained skins were compared in their properties. The results are given in Table 2, below.

TABLE 2

Properties		Contro 1	Protease HY- 3	Amoro n	Oropon K
Shrinkage (%)	Width	20	18	17	20
	Length	19	19	19	22
Softness (mm)		1.9	2.6	2.8	2.1
Tensile Strength (kgf/mm ²)		0.7	0.8	0.8	0.6
Tear strength (kgf/mm ²)		2.3	2.1	2.4	1.8
Elongation (%)		64	105	89	70

1-2 Addition of Ammonium Sulfate

While performing the same procedure as in the above
 5 table 1, 3 wt% ammonium sulfate ((NH₄)₂SO₄) was added at the
 bating step and stirred for 60 minutes. Additionally,
 protease HY-3, Amoron and Oropon K were used. The raw
 skins were cut to width 30 cm x length 20 cm. As a control,
 the no deliming agent was used in the bating step.

10 Thusly obtained skins were compared in their
 properties. The results are given in Table 3, below.

TABLE 3

Properties		Contro 1	Protease HY- 3	Amoro n	Oropon K
Shrinkage (%)	Width	23	16	24	23
	Length	23	18	22	22
Softness (mm)		1.7	2.4	2.2	2.1
Tensile Strength (kgf/mm ²)		0.8	0.8	0.9	0.9
Tear strength (kgf/mm ²)		2.4	1.6	1.4	2.1
Elongation (%)		43	77	76	77

15

1-3 Addition of Ammonium Chloride and Ammonium Sulfate

While performing the same procedure as in the above

table 1, 1 wt% ammonium chloride (NH_4Cl) and 2 wt% ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) were added in the bating step and stirred for 60 minutes. Also, protease HY-3, Amoron and Oropon K were used. The raw skins were cut to width 30 cm x length 20 cm. As a control, no deliming agent was used in the bating step.

Thusly obtained skins were compared in their properties. The results are given in Table 4, below.

TABLE 4

Properties		Contro 1	Protease HY- 3	Amoro n	Oropon K
Shrinkage (%)	Width	16	19	17	19
	Length	26	20	25	28
Softness (mm)		3.1	3.8	3.3	3.2
Tensile Strength (kgf/mm ²)		1.4	1.4	1.4	1.2
Tear strength (kgf/mm ²)		2.3	2.4	2.5	2.2
Elongation (%)		121	138	113	107

As stated above, the surface of skins after ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) was used alone in the bating step was similar to that of skin surfaces after Amoron and Oropon K were used. When ammonium chloride (NH_4Cl) alone and a mixture of ammonium chloride (NH_4Cl) and ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) were used, the elongation percentage was excellent. The skins had more even surface and clearer grain, compared to Amoron and Oropon K. In terms of shrinkage and softness, the results were similar.

When the protease HY-3 was used only with ammonium

sulfate, moderate effects were seen, and the synergic effects were seen when ammonium chloride alone or a mixture of ammonium chloride and ammonium sulfate were added.

5 COMPARATIVE EXAMPLE 2 : Measurement of Properties of Practically Used Skin

A piece of skin having a thickness of 1.2-1.4 mm, subjected to liming step, was divided into two pieces, one
 10 of which was added with protease HY-3 and the other of which was added with an imported bating agent. Then, bating was conducted. The skins were made to crust (that is, subjected to a tanning process to prepare skins ready for making leather goods (e.g., handbag and shoes)), which
 15 was then subjected to the method as in the following table 5. The surface state of skins was observed and physical strength of skins was measured.

TABLE 5

Step	Chemicals	%	Time (min)	PH
Delimin g	H ₂ O (30 °C)	20 0		
	Deslon/NAT (TEXAPEL)	0. 2		
	(NH ₄) ₂ SO ₄	2		
	NH ₄ Cl	1	60	7. 6
Bating	Lactic acid	0. 3	20	
	Bating Agent	0. 8	60	
	Dry			

When the protease HY-3 and the imported Oropon K were used, the properties for skins are shown in Table 6, below.

5

TABLE 6

Agent		Protease HY-3	Oropon K
Thickness		1.2-1.4	1.2-1.4
Tensile Strength (kg/mm ²)	Head	2.7	1.1
	Medium	2.5	2.4
	Terminal	2.5	1.9
Tear Strength (kg/mm ²)	Head	5.8	4.1
	Medium	5.1	5.1
	Terminal	7.3	5.8
Bursting Strength (kg/mm ²)	Head	40 or more	32
	Medium	40 or more	40 or more
	Terminal	40 or more	37
Elongation (%)	Head	76	59
	Medium	77	82
	Terminal	60	70
Softness (mm)	Head	3.9	4.2
	Terminal	3.9	3.7
	Medium	3.3	3.5

From the results of the above table 6, it can be seen that the protease HY-3 is more excellent in almost all properties than the imported Oropon K. In the tensile strength, the protease HY-3 treated leather was 2.5-2.7 kg/mm² while the Oropon K treated leather was 1.1-2.4 kg/mm². In the tear strength, the former was 5.1-7.3 kg/mm² while the latter was 4.1-5.8 kg/mm². Also, in the bursting strength, the former was 40 kg/mm² or larger while the latter was 32-40 kg/mm². As for elongation percentage and softness, Oropon K treated leather was shown as 59-82 % and 3.5-4.2 %, whereas the protease HY-3 of the present

invention produced leather of 60-77 % elongation percentage and 3.3-3.9 % softness.

COMPARATIVE EXAMPLE 3 : Protein Decomposition by Protease

5 **HY-3**

In order to compare protein decomposition by protease HY-3 produced from *Aranicola proteolyticus* HY-3 strain (KCTC 0268BP) with that of other protease, the protease HY-
10 3 of the present invention and the imported Oropon K were used. Oropon K, as a pancreatic enzyme preparation, is widely used for increasing tensile strength and softness of grain and maintaining soft grain by decomposing collagen without damaging the grain of leather.

15 The activity of protease HY-3 was measured by Braun, V. & Schmitz, G., Arch, Microbiol. 1980, 124: 55-61. 0.24 g of azo-casein (Sigma, USA) was dissolved in 10 ml of 50 mM phosphate buffer, pH 7.5, to prepare a substrate solution. 300 µl of substrate solution was mixed with 100 µl of
20 culture medium and reacted at 37 °C for 30 minutes. To the reaction, 300 µl of 10 % trichloroacetate was added to further react at room temperature for 1 hour. The resulting reaction was centrifuged at 7,000 rpm and the pellet was separated from the supernatant. 300 µl of supernatant was
25 added with 30 µl of 10 % sodium hydroxide and then absorbance was measured at 420 nm. 1 unit of enzyme of the present invention was defined as the amount of enzyme

releasing an amount of azo and casein, sufficient to increase absorbance by 1.0 after 1 minute of digestion of azo-casein test substrate, at 37 °C.

1 unit of each of protease HY-3 and Oropon K was added to a protein substrate mixture (1 mg/ml) of casein, albumin, hemoglobin and keratin, and a protein substrate mixture (5 mg/ml) of collagen and elastin, and then reacted at 37 °C for 2 hours. Thereafter, using a Bradford method, the amount of protein in samples was measured (see, Table 7). Then, 1 unit of enzyme was defined as the amount required to produce 1 µg protein equivalent from proteolytic digestion of the substrate at 37 °C for 1 minute.

Accordingly, the protease HY-3 of the present invention shows higher decomposition activity against most substrates, exclusive of hemoglobin, than Oropon K. Generally, animal skins comprise structural proteins of collagen, elastin, keratin and so on, and non-structural proteins of albumin, globulin and the like. The protease HY-3 can decompose casein and albumin, which are also present in skins, and in particular, has excellent decomposition activity against keratin, which is a main component of hairs, so that it consequently functions in the liming and deliming steps.

Through digestion for 10 minutes by the protease, keratin and collagen can be decomposed by 40 % or more. The results of the decomposition for proteins excluding hemoglobin by the protease HY-3 are presented in Table 7,

below.

TABLE 7

Protein	Relative Decomposition (%)							
	Protease HY-3				Oropon K			
	10 min.	30 min.	1 h.	2 h.	10 min.	30 min.	1 h.	2 h.
Casein	93	94	95	100	65	70	73	85
Albumin	10	14	15	44	0	10	15	17
Hemoglobi n	2	4	12	16	0	5	11	17
Keratin	37	40	40	46	17	18	24	34
Collagen	34	63	66	98	2	15	27	41
Elastin	4	13	18	21	1	4	19	20

5

In addition, the protease HY-3 was 4 times more active against collagen, compared to Oropon K. The results are shown in Table 8, below. The structural proteins, constructing most animal skins, constituting collagen of about 60 % or more, and collagen is further present in bones, muscles and tendons. So, it is believed that, if a given protease can effectively decompose collagen, it has excellent effect for leather processing.

15

TABLE 8

Protein	Enzyme Activity (unit/ml)	
	Oropon K	Protease HY-3
Casein	10.9	14.8
Albumin	1.6	2.2
Hemoglobin	0.8	0.6
Keratin	2.8	6.2
Collagen	2.3	9.8
Elastin	0.7	2.0

INDUSTRIAL APPLICABILITY

As described above, through the method for preparing leather using protease HY-3 and the method for treating wastes derived from leather processing of the present invention, excellent quality of leather can be obtained and also amounts of chemicals used in leather processing can be drastically decreased, thereby treating or recycling wastes in an environmentally friendly manner.

10 The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

What is claimed is:

1. A method for preparing leather, comprising the steps of soaking, liming, deliming and bating, wherein
5 protease HY-3 produced by *Aranicola proteolyticus* HY-3 strain (KCTC 0268BP) is added to the soaking step, the liming step, the deliming step or the bating step.

2. The method as set forth in claim 1, wherein the
10 protease HY-3 is a microorganism-removed enzyme-containing liquid from said microorganism culture medium, or a formulated preparation.

3. The method as set forth in claim 1, wherein the
15 protease HY-3 is used at the amount of 0.1-15 wt%.

4. A method for treating wastes derived from leather preparation, wherein protease HY-3 produced by *Aranicola proteolyticus* HY-3 strain (KCTC 0268BP) is added to
20 wastewater and solid wastes derived from the soaking step and the liming step, solid wastes derived from the liming and deliming steps and the bating step, or solid wastes from a finishing step.

25 5. The method as set forth in claim 4, wherein the protease HY-3 is a said microorganism culture medium, a microorganism-removed enzyme-containing liquid from said

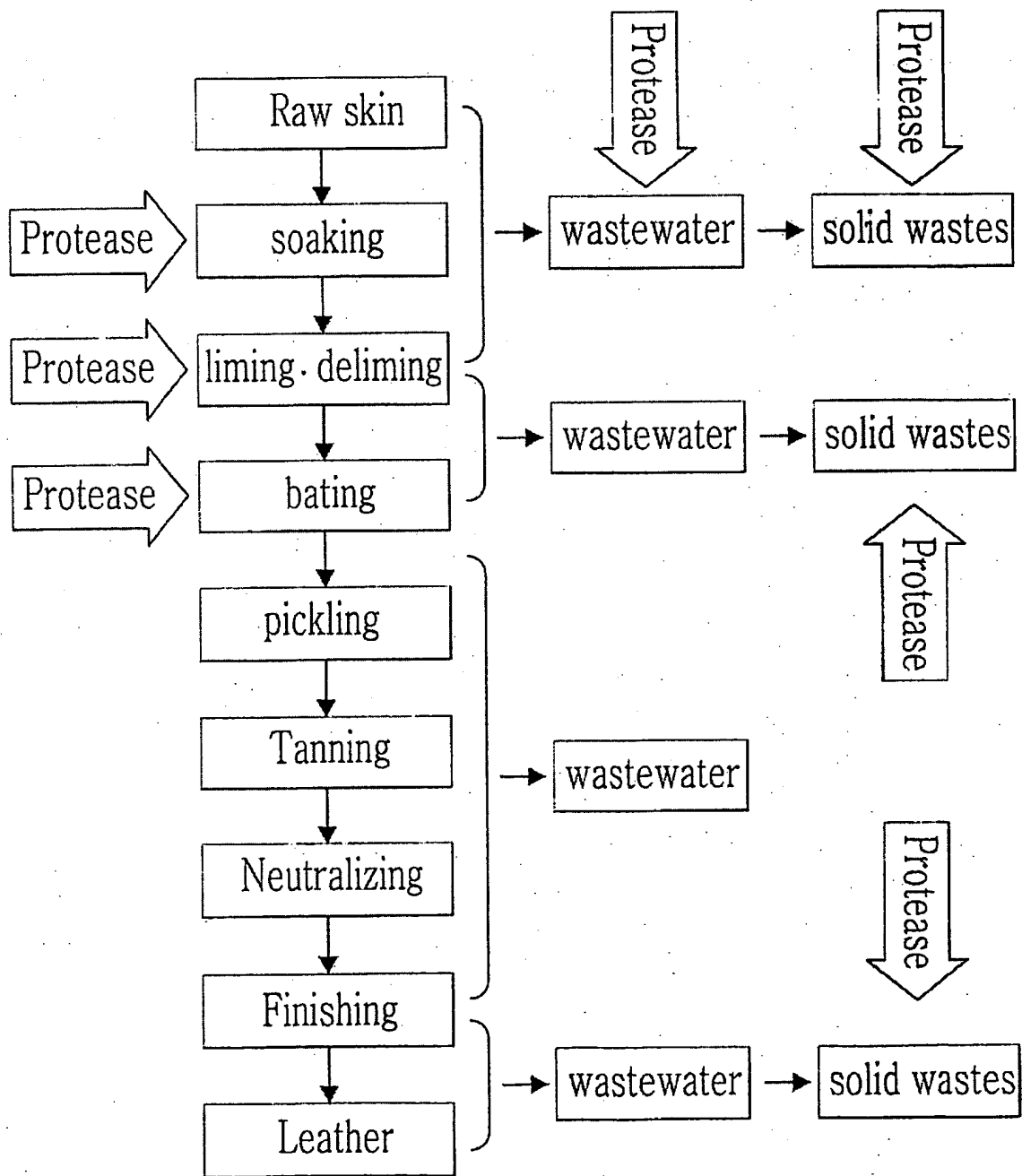
medium, or a formulated preparation.

6. The method of claim 4, wherein the protease HY-3 is
added in combination one selected from the group consisting
5 of lipase, amylase and mixtures thereof.

1/2

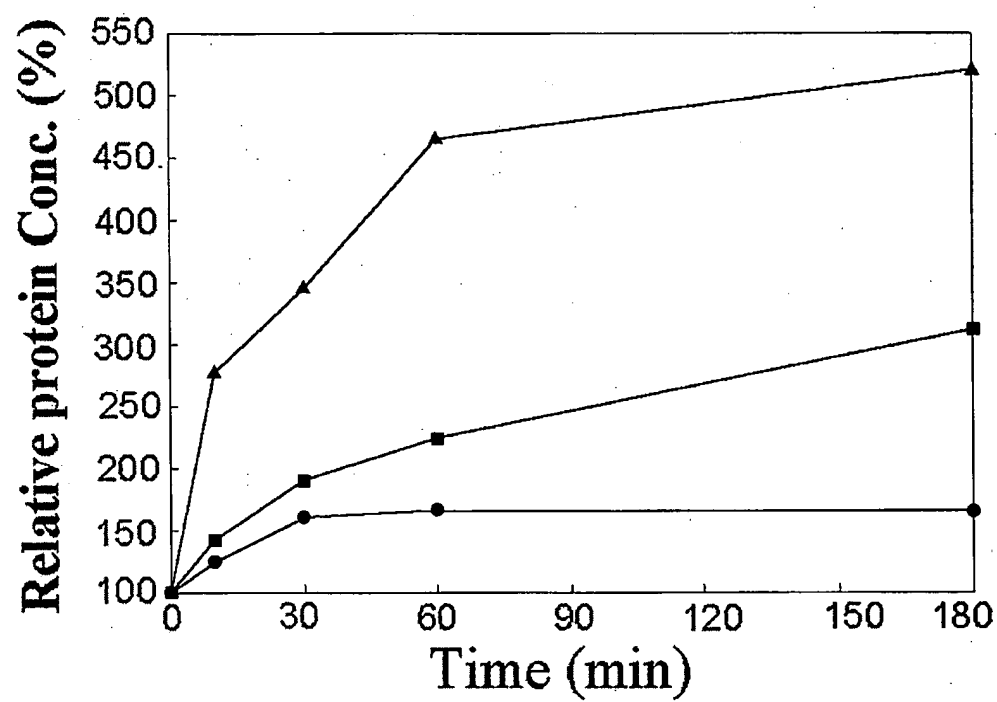
Figures

FIG. 1



2/2

FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR01/01942

A. CLASSIFICATION OF SUBJECT MATTER		
IPC7 C14C 3/28		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N 9/50, C14B 1/00, 1/20, 7/00, C14C 1/00, 1/04, 1/06		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Korean Patents and applications for inventions since 1975 Korean Utility models and applications for Utility models since 1975		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) NPS, PAJ		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP A 7-118700 (KOOKEN KAGAKU KK.) 9. MAY 1995	1
A	JP A 3-124800 (SHOWA DENKO KK.) 28. MAY 1991	1
A	WO A1 96/11285 (NOVO NORDISK A/S) 18. APRIL 1996	1
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search 22 APRIL 2002 (22.04.2002)		Date of mailing of the international search report 23 APRIL 2002 (23.04.2002)
Name and mailing address of the ISA/KR Korean Intellectual Property Office Government Complex-Daejeon, 920 Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140		Authorized officer CHOI, Seung Keun Telephone No. 82-42-481-5575



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR01/01942

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 7-118700 A	09. 05. 1995	None	
JP 3-124800 A	28. 05. 1991	None	
WO 96/11285 A1	18. 04. 1996	DE 69513676 T2	06.07.2000
		EP 784706 B1	01.12.1999
		AU 696441 B2	10.09.1998
		JP 10-506944 T2	07.07.1998
		CN 1176664 A	18.03.1998